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Patterning Lipid Bilayers on Microfabricated Metal Electrodes

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Lipid molecules were immobilized on the surface of photolithographically patterned chromium and titanium. Large unilamellar lipid vesicles were found to bind on the native oxide surface of patterned support metals. Metal evaporation and resist liftoff techniques were used to pattern metal on a hydrophobic polymer surface. Lipids bound on solid substrates provide a biological interface for current measuring electrodes to detect bound cell or biomaterial. This patterning technique provides means to specifically bind lipids and conjugated biomaterials (polyethelene glycol (PEG), biotin, fluorescence dyes, and DNA oligimers) to the electrode surface. This technique may be applied to patterning biomaterial on metal inside thermally bonded microfluidic channels, to form titanium coated biomedical implants, and to create robust lipid-conjugated electrodes for biosensor applications.

INTRODUCTION

Supported lipid bilayers provide an excellent model to analyzing the biological processes of the cell membrane.[1] Lipids are the primary constituents of biological cell membranes, making up 22-79% of the membrane molecules.[2] Over the past two decades, lipids have been patterned on silicon wafer substrate in studies referred to as "black lipid" membrane patterning.[3] Lipid patterning can be performed through several different microfabrication techniques. μCP techniques have been used to create a geographic protein barriers for the lipids, referred to as "corrals".[4,5] Similarly, a 10 nm thick gold corrals have been patterned on silicon creating lipid islands.[6] Electric fields were used to induce concentration gradients in lipid bilayers.[7] These papers have used photobleaching experiments and scratching studies to demonstrate the fluidic nature of the top layer of the lipid bilayer. These technologies utilize the property of the lipids nonspecifically binding native oxide on the silicon substrate surface. Another study used

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[1] Brian, A; McConnell, H. M. *Proc. Natl. Acad. Sci. U.S.A.* **1984**, *81*, 6159.
[2] Guidotti, G. 1972 **An. Rev. Biochem.** and Lotan, R. and Nicholson, G. L. in **Advanced Cell Biology** ed. by L. M. Schwartz and M. M. Azar. Van Nostrand (New York; 1981).
[3] Hanke, W.; Schluhe, W.-R. *Planar lipid bilayers. Methods and applications;* Academic Press Limited: London, 1993.
[4] Hovis, J.; Boxer, S. *Langmuir* **2000**, *16*, 894-897.
[5] Kung, L. A.; Kam, L.; Hovis, J.; Boxer, S. *Langmuir* **2000**, *16*, 6773-6776.
[6] Grooves, J. T.; Ulman, H.; Boxer, S. G. *Science* **1997**, *275*, 651-653.
[7] Grooves, J. T.; Boxer, S. G. *Biophysical Journal* **1995**, *69*, 1972-1975.

spectral neutron reflection to characterize the structure of single lipid bilayers adsorbed to silicon surface from aqueous solution and confirmed adsorption with AFM analysis.[8]

This research illustrates how high-resolution micron-scale patterning methods have been developed to immobilize functional proteins on a silicon dioxide substrates for biosensor applications. Micro- and nanofabrication techniques have allowed the immobilization accuracy to reach resolution at a sub-micron level with repeatable and accurate capability. This has enhanced the ability to analyze and measure the processes at the cellular membrane level. Many patterning techniques use lithographic methods, most often borrowed from microelectronics technology, to reproduce a mask pattern using biologically relevant chemicals.[9] Photopatterning has been used to spatially distribute biomolecules, such as enzymes, antibodies, and nucleic acids, which are used in the development of biochips.[10] Microcontact printing (μCP) is a versatile method for patterning micrometer size patterns on silicon, glass, or plastic substrates. Antibodies have been patterned to 1 μm resolution with μCP. Additional commonly used protein patterning techniques include inkjet printing, micromolding, microfluidic network flowing of biomaterials onto silicon or glass substrates.

In this paper, we demonstrate a photolithographic method for patterning lipids on a hydrophobic polymer surface. An acetone resistant polymer, Zeonor 1020, was processed with a metal lift-off technique to form micrometer sized metal patterns on its surface. The patterned polymer substrate was covered with a solution containing 100 nm unilamellar lipid vesicles comprising a lipid-conjugated fluorophore for visualization using epifluorescence microscopy. Following a brief 5-minute exposure, the lipid vesicles adhered to the metal surface creating a metal-lipid bond that was not removed by subsequent washing steps. Thus, this technique offers a means to immobilize biomolecules on a metal-patterned solid planar substrate or inside microfluidic channels formed from metal-patterned, thermally bonded plastic substrates. Thus, a novel technique is presented for patterning lipids and lipid-conjugated molecules onto a plastic substrate. Future applications include using microfabricated polymer structures coated with titanium and lipids for biomedical implant applications.

EXPERIMENTAL SECTION

Reagents. Vesicles were prepared with 1-palmitoyl 2-oleoyl phosphatidylcholine (POPC) lipids and rhodamine-conjugated dioleoylephosphatidylethanolamine (Rh-PE), both from Avanti Polar Lipids (Alabaster, Alabama). Zeonor 1020 polymer was used as the substrate (Zeon Chemicals, Tokyo, Japan).

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[8] Koenig, B. W.; Krueger, S.; Orts, W. J.; Majkrzak, C. F.; Berk, N. F.; Silverton, J. V.; and Gawrisch, K. *Langmuir* **2000**, *12*, 1343-1350.

[9] Branch, D.W.; Wheeler, B. C.; Brewer, G J.; Leckband, D.E. **2000**. *IEEE Transactions on Biomedical Engineering 47*, 290-300.

[10] Dontha, N.; Nowall, W. B.; Kuhr, W. G.; *Anal. Chem.* **1997**, 69, 2619.

Tap water was filtered to a resistivity of 18.2 Mohm-cm using a Milli-Q Millipore filtration system. Microposit MIF 300 developing solution was used to develop the exposed 5 μm thick Shipley 1805 positive photoresist. 1" plastic Petri dishes were purchased from Fischer Chemicals (Pittsburgh, PA). Polyclonal, goat anti-mouse IgG antibodies and biotinylated goat anti-*E.coli* O157:H7 antibodies were purchased from Kirkegaard & Perry Laboratories (KPL, Gaithersburg, MD). N-[2-Hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]) (HEPES), sodium chloride (NaCl), and phosphate buffered saline (PBS) were purchased from Aldrich (Milwaukee, WI). 0.1 μm Nucleopore, polycarbonate filters, were purchased from Whatman, Incorporated (Clifton, NJ).

Development of Microfabricated Pattern. A 4" chrome plated quartz mask was processed in the GCA PG3600F Optical Pattern Generator using a pattern designed with L-Edit software. This mask was developed in a chrome etchant for 2 minutes, washed with deionized (DI) water, and developed in MIF 300 for 2 minutes.

Photolithography. 500 nm of Shipley 1805 was coated onto Zeonor and silicon wafers at 3000 rpm for 60 seconds and a 2-second accelcration/deceleration. The samples were pre-baked for 2 minutes in 90°C oven. The samples were exposed using the GCA 6300 DSW Projection Mask Aligner and the HTG System 3HR Contact/Proximity Aligner with the quartz patterned mask. The samples were exposed with UV light at 365 nm for 0.8 seconds at a 15 mW/cm^2 intensity. The samples were developed in MIF 300 for 30 seconds, washed in DI water for 2 minutes, and dried in a nitrogen gas stream. The samples were post-baked for 2 minutes in 90°C oven. 20 nm of metal was evaporated onto the samples using either the CHA RAP-600 Thermal Evaporator (chromium) or the CVC SC4500 Combination Thermal/E-gun Evaporation System (titanium). The samples were washed in a sonication acetone bath for 3 seconds, and immersed in acetone for 5 minutes. The samples were dried with a nitrogen gas stream.

Lipid Preparation. Lipids were dissolved in chloroform and stored at -20°C. A 25 mM/ml solution of POPC/Rh-PE (99:1 molar ratio) was prepared in $CHCl_3$. 5 μmoles of total lipid was transferred to 13x100 mm glass test tubes and dried with a stream of nitrogen gas. These test tubes were then dried further under 10^{-5} torr of vacuum for 1 hour. The solution was rehydrated to a final lipid content of 5 mM using 150 mM NaCl, 10 mM HEPES, pH 7.4 to remove residual organic solvent. The solutions were vortexed and subjected to 5 freeze-thaw cycles (N_2/room temperature water). The vesicle solution was extruded 10X as described by Mayer using two stacked 0.1 μm pore polycarbonate filters using a high pressure 10 mL Thermobarrel Extruder from Northern Lipids (Vancouver, British Columbia). Lipid vesicles were diluted with PBS as desired.

Lipid Application. 30 μL of a 1 mM lipid solution was applied onto the metal-patterned polymer substrate under yellow light (UV-safe) conditions. After a 5-minute lipid exposure, the sample was immersed in Millipore filtered water for 1 minute. The sample was transferred to a second Millipore filtered water beaker in a 1" Petri dish and immersed for 1 minute. A cover slip was applied onto the sample surface under water. The sample, with cover slip attached, was removed from the water and analyzed using a in a Zeiss upright microscope. An Omega Optical XF 102 rhodamine fluorescent filter set (590 nm/619 nm excitation/emission) was used to analyze the samples. Images were captured using a Spot camera (Diagnostic Instruments, Inc., Sterling Heights, MI) and a 600 MHz P3 PC (using Spot Imaging Software version 2.2.2).

RESULTS AND DISCUSSION

Shipley 1805 photoresist adhered to the Zeonor polymer and provided sufficient aspect ratio for an effective metal liftoff process. 30 second development of the photoresist was sufficient, while over 1 minute would overdevelop the pattern. The pre- and post-bake enhanced the stability of the exposed resist. A 3-second sonication adequately removed the metal during the liftoff process.

The lipids were easily integrated into the experiment. Lipid vesicles containing 1 mol% Rh-PE (negatively charged) provided sufficient fluorescence to generate vivid images using epifluorescence microscopy. The POPC lipids is neutral in charge and spontaneously bound to the oxidized metal surface rapidly after application.

The μCP process for applying lipids as detailed by Boxer offered an effective technique to spatially immobilize lipids, as illustrated in Figure 1.[11] The lipids used were POPC/Rh-PE (99/1) lipid (25 μmoles). The dark areas in this picture are 25 μm lines of anti-*E.coli* antibodies. The antibodies provide a barrier to lateral diffusion of lipids.

The patterning with lipids bound to evaporated metal oxide offers a permanent substrate onto which the lipids can preferentially adhere. Figure 2a is a micrograph of lipids patterned on Zeonor polymer of 25 μm chromium lines. Figure 2b is the bright field image of the same sample illustrating the localization of the lipids bound to the metal. These pictures demonstrate the high specificity of the lipids for the metal compared to the minimal binding to the unpatterned, bare plastic (black). Figure 3 illustrates a higher magnification of the lipid patterned to the 25 μm chromium-sputtered lines. Figures 4 and 5 illustrate images of lipids binding to patterned titanium oxide in a similar manner as the lipids binding to the chromium oxide in figures 2a, 2b and 3.

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[11] Hovis, J.; Boxer, S. *Langmuir* **2000**, *16*, 894-897.

The patterning of POPC/RE-PE lipids in this experiment utilized the hydrogen bonding between the glycerol head group and the metal oxide. The high resolution attained from the nanofabrication technology coupled with the high selectivity of the lipids for the oxidized metals offers an easy and reliable method for patterning lipids in spatially defined areas. This technique offers a one step approach to applying lipids and molecules conjugated to lipids onto the patterned, metal surface. Second, when compared to a silicon surface the intrinsic hydrophobic nature of the polymer reduces the need for surfactants and other surface treatments (i.e. gold-thiol-PEG coating) to minimize nonspecific binding. Third, this is a rapid process requiring less than 5 minutes for the lipid application.

Zeonor 1020 had several advantages over other polymers and plastics. It is a plastic that is resistant to degradation in acetone, thus allowing the metal liftoff step to be performed with acetone that rapidly dissolves exposed photoresist. The low glass transition temperature of Zeonor 1020 allowed low temperature thermal bonding (90°C) to be performed.[12] A 1 square inch piece of Zeonor 1020 is low cost, approximately $0.10/trial. The high hydrophobicity resisted nonspecific binding on areas other than on the metal; consequently, there was a high signal/noise ratio, where the binding of the lipid is negligible and below detection. The oxidized metal surfaces offered a hydrophilic substrate onto which lipids bound. Additionally, the lipid bilayer offered a biocompatable foundation onto which or into which other molecules could adhere.

CONCLUSION

Lipid application onto photolithographically patterned metals is a rapid and economical way to transfer micrometer scale patterns to a polymer surface. Future applications for this technology include patterning a variety of lipids on the metal surface, creating biocompatable implants, performing impedance measurements for biosensor applications and forming electrodes the lipids bind onto. Potential applications include different lipid-conjugated molecules, including polyethelene glycol (PEG), biotin, avidin, fluorescence, polysaccharides, and oligonucleotides. Electrodes can be formed by using a gold layer patterned under the titanium layer would offer to electrode conductivity. These electrodes could have the lipids adhere, and form an interface for other biological media to attach on the surface, creating lipid/titanium coated polymer implants. The application of lipids over the titanium layer can modulate the biological interactions occurring at the interface for a biomedical implant. PEG binding to the lipids can create areas resistant to molecular binding and avidin-conjugated lipids can promote binding of biotinylated molecules to the lipid interface. Furthermore, smaller features can be achieved using a deep UV contact aligner and 10X stepper to obtain a 1 μm and 500 nm feature resolution, respectively.

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[12] Kameoka, J.; Craighead, H. G. *Anal. Chem.* **2001**, XXX, XXX.

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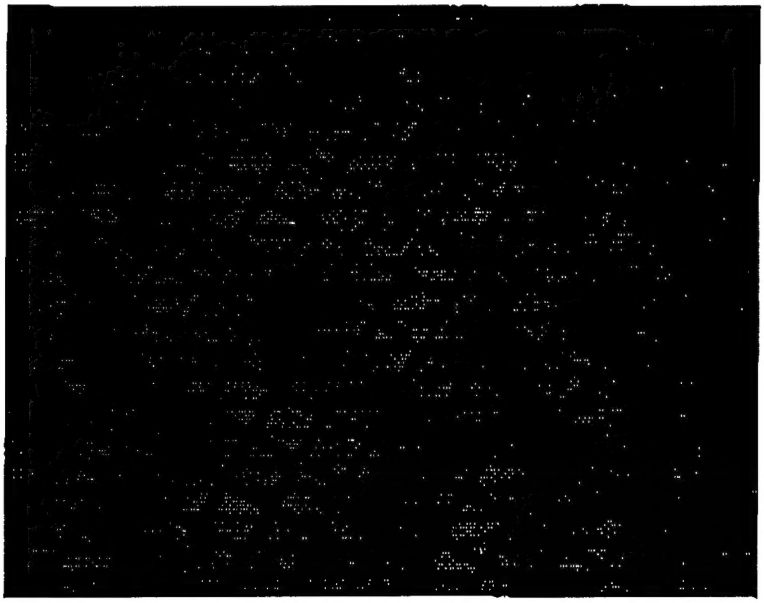

Figure 1. Optical micrograph of 25 μm lines of anti-*E.coli* antibodies (black) patterned using the μCP process with 75 μm squares of POPC-/Rh-PE (99/1) lipid (25 μmoles).

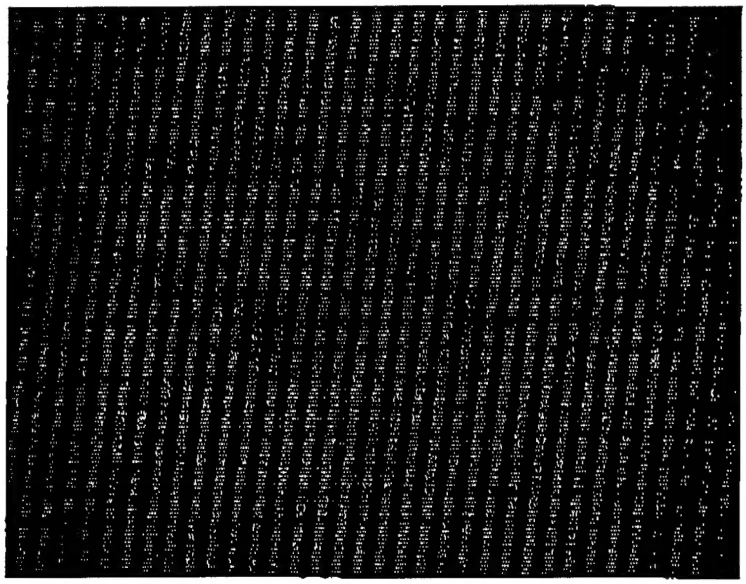

Figure 2a. Fluorescent image of 25 μm lines of POPC-/Rh-PE (99/1) lipid (25 μmoles) bound to the patterned 20 nm thick evaporated chromium oxide lines.

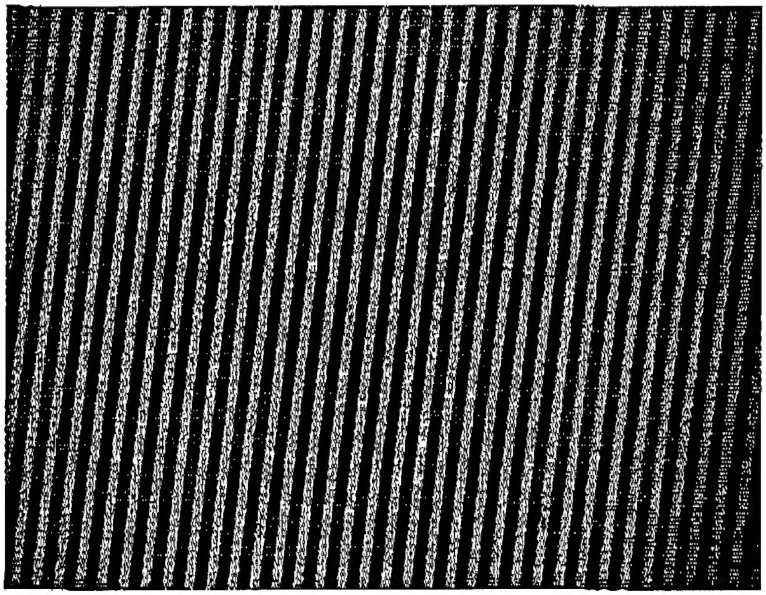

Figure 2b. Bright field image of 25 μm lines of POPC-/Rh-PE (99/1) lipid (25 μmoles) bound to the patterned 20 nm thick evaporated chromium oxide lines.

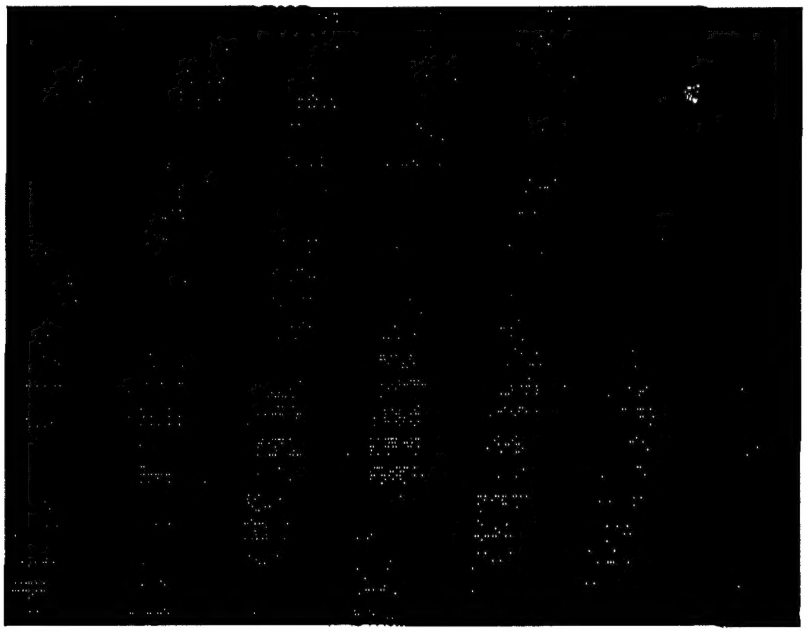

Figure 3. Fluorescent image of 25 μm lines of POPC-/Rh-PE (99/1) lipid (25 μmoles) bound to the patterned 20 nm thick evaporated chrome lines.

Figure 4. Fluorescent image of POPC-/Rh-PE (99/1) lipid (25 μmoles) bound to the patterned 20 nm thick evaporated titanium with a 100 μm line (black) separation.

Figure 5. Fluorescent image of POPC-/Rh-PE (99/1) lipid (25 μmoles) bound to the patterned 20 nm thick evaporated titanium with 40 μm line (black) separation and 100 μm node.

Conklin, in *Avidin-Biotin Chemistry: A Handbook*, p 76, (Pierce Chemical Company, Rockford, 1994).

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